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13. ABSTRACT (Maximum 200 words)

The aims of this research were to study each of the various molecular mechanisms whereby toxic metal cations and oxyanions are immobilized by bacteria that live in the soil. The research effort focussed on two bacteria: *Xanthomonas maltophilia* strain OR-02, an organism that chemically transformed individual metals to a less mobile state; and *Pseudomonas mendocina* strain AS302, a bacterium that formed tight complexes with a wide variety of toxic metal ions. The NADPH-dependent reduction of Hg(II) to elemental mercury by OR-02 was catalyzed by an inducible mercuric reductase. The reduction of selenite and tellurite to their insoluble elemental forms was mediated by an intracellular glutathione reductase that utilized the spontaneously-formed bis(glutathio)Se or bis(glutathio)Te, respectively, as pseudosubstrates. The 3-electron reduction of hexavalent chromium was catalyzed by a membrane-bound chromate reductase. A total of 14 different heavy metal ions were shown to bind tightly to strain AS302 with biosorption capacities that ranged from 320 (Pb) to 680 (Ag) μmol metal/g dry cell weight. This project could provide useful information toward the eventual exploitation of these organisms for the removal of toxic metal wastes from selected, heavily polluted sites.

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FINAL TECHNICAL REPORT

May 1, 1992 - April 30,1995

AFOSR Contract F49620-92-J-0246: Biotransformation of Toxic Metals by Bacteria

OVERVIEW

The overall aims of this project were to study each of the various molecular mechanisms whereby toxic metal cations and oxyanions were immobilized by two remarkable strains of bacteria: *Xanthomonas maltophilia* strain OR-02; and *Pseudomonas mendocina* strain AS302. The specific aims for the previous grant period were as follows:

(1) To perform detailed kinetic studies on the transformation and immobilization of soluble lead, chromium, and other metals as catalyzed by strains OR-02 and AS302. The purpose of these experiments was two-fold: (i) to identify the optimum solution conditions necessary to achieve maximal and timely removal of each soluble metal species; and (ii) to identify and document potential sources of interference for each metal transformation activity. The latter experiments focused on the possible inhibition of metal transformations by metal-complexing agents and/or other metal cations and oxyanions;

(2) To investigate various means for immobilization of either strain and to determine the efficacy of the immobilized bacterium for the removal of soluble metals from contaminated groundwater. Immobilization strategies included, but were not limited to, entrapment within calcium-alginate beads and adsorption of the live bacteria onto a hydrophobic, porous polyurethane; and

(3) To identify, separate, purify, and reconstitute the minimum cellular components necessary for metal transformation in strain OR-02.

RESEARCH ACCOMPLISHMENTS

Kinetic studies with intact bacterial cells

When *X. maltophilia* strain OR-02 was cultured in the presence of each of 8 different soluble metal species, growth of the organism was accompanied by the disappearance of the soluble metal species from solution, along with the concomitant appearance of an insoluble form of the metal.

(i) Optimization of Pb and Cr transformation with strain OR-02 - When naive cells of strain OR-02 were cultured in the presence of 1.0 mM Pb(II), growth of the organism was accompanied by the disappearance of the soluble Pb(II) from solution. The missing lead was all contained in small brown-black biocolloids from 50 to 150 nanometers in diameter (by contrast, the intact bacterial cells were rod-shaped with a diameter of 0.5 to 1.0 micrometers and a length of approximately 3 to 5 micrometers). The lead biocolloids bore considerable negative charge on the surface of the particle, as evinced by the 30 mV of zeta potential on the colloid revealed by electrophoretic mobility measurements.

The disappearance of soluble lead from spiked cultures of OR-02 was quantified by analyses of aliquots from the culture on a Perkin-Elmer 2100 atomic absorption spectrophotometer. Both the temperature and pH of the culture influenced the growth of the organism and the concomitant disappearance of soluble lead. The optimum pH for both growth of the organism and the removal of soluble lead was 7.5. However, temperature optima for growth of the bacteria and the removal of soluble lead were different, being 35 and 20° C, respectively. Above about 30° C, lead immobilization did not occur, even though growth of the organism was abundant.

The exact formulation of the culture medium under otherwise optimal conditions had no quantifiable influence on the disappearance of soluble lead. Thus the timely bacterial-dependent immobilization of soluble lead occurred equally well in both minimal (glucose plus mineral salts) and complex (tryptone plus yeast extract) media, even though components of the latter formed tight complexes with the lead cation.

Experiments were initiated to investigate whether the immobilized bacterium could be exploited for the removal of soluble lead. Protocols were developed to entrap and immobilize strain OR-02 in calcium-alginate beads. The efficacy of lead removal by immobilized bacteria was examined in a stirred-tank reactor for the following 3 preparations: (i) bacteria were entrapped in beads in the presence of soluble lead; (ii) beads were formed in the presence of lead

followed by the adhesion of bacteria to the preformed beads; and (iii) bacteria were entrapped in beads and then exposed to soluble lead. Only the latter preparation was effective in the removal of lead from solution. The beads containing immobilized bacterial-lead complexes were readily separated from the remediated solution by filtration.

Growth of strain OR-02 in the presence of chromate was accompanied by the gradual (24 to 48 hr) electrochemical reduction of the yellow Cr(VI) (chromate) to the faint blue-green trivalent Cr(III). Strain OR-02 grew after a 2-hr lag period when inoculated into rich media containing up to 1.0 mM chromate. Optimal culture conditions for the bacterial-dependent reduction of Cr(VI) were identical to those for the corresponding immobilization of lead: rich media (Luria broth); pH 7.5; and 25° C.

Although bacterial growth was severely inhibited at 5.0 mM chromate, biological reduction of the chromate was still observed. Indeed, effective reduction of up to 30 mM chromate was readily observed with cell suspensions of strain OR-02, although the bacterium did not grow under those conditions.

(ii) Optimization of citrate extraction of lead in soil samples - New methods were sought to enable investigators to extract heavy metals from soil and sediment samples without recourse to strong acids, high temperatures, or cumbersome equipment. Empirical optimization indicated that a 15 minute incubation with 10 mM citrate at pH 7.5 extracted an average of 50% of the total lead from 12 dried samples derived from spoilbanks along a metal-contaminated bayou in southern Louisiana. The concentration of citrate used in these experiments is compatible with the culture conditions for strain OR-02 on citrate as the sole carbon and energy source.

(iii) Optimization of bacterial degradation of citrate-metal complexes - Citrate forms a tight 1:1 complex with Pb(II). Culture of strain OR-02 in the presence of equimolar concentrations of lead and citrate produced the same level of lead biocolloid formation as that observed in the absence of citrate. However, an increasing molar excess of citrate in the culture medium served to inhibit both the rate and the extent of lead biocolloid formation; only about 20% of the metal in a 1.0 mM solution of lead was immobilized in media amended with 10 mM citrate. Efforts to optimize lead biocolloid formation in the presence of concentrations of citrate in excess to that of Pb(II) were inconclusive.

(iv) Characterization of metal-laden biocolloids - This laboratory has always maintained an overt, if not a covert, dissatisfaction with the lack of quantitative characterization of the biocolloids produced by strain OR-02 in the presence of selenite, lead, bismuth, etc. The disappearance of soluble metal may be readily quantified, but the metal-laden colloids themselves have remained poorly characterized. Accordingly, one focus during the current grant period was to investigate whether instrumentation commonly employed to characterize the interactions and stability of colloidal particles could be exploited to investigate the biocolloids produced by *X.*

maltophilia in the presence of selenite, lead, and other soluble toxic metal species. The biocolloids produced by *X. maltophilia* in the presence of either Pb(II) or Se(IV) were characterized by both static light scattering and electrical impedance methods. Results from both methods indicated that the biocolloids resembled cells in size and shape. Transmission electron microscopic studies revealed that, unlike intact cells, the biocolloids were comprised of large, electron-dense bodies that contained the majority of the metal ion sequestered by the culture. Complete separation of these biocolloids from intact cells was accomplished by sedimentation field flow fractionation. Now that a means is available to physically separate metal-laden cells from metal-free cells, a number of interesting questions can be posed. For example, why do some cells in a presumably homogeneous population of cells derived from a single colony accumulate visible quantities of metals while others do not?

In addition, we discovered that the OR-02 appeared to express an organolead lyase activity. When grown in the presence of up to 1.0 mM mono-, di-, or triethyllead chloride, OR-02 generated the same black precipitate as that obtained in the presence of mere inorganic Pb(II). Such an organolead lyase activity has never been described in the literature.

(v) Optimization of metals immobilization by strain AS302 - Growth of this organism in rich media in the presence of a variety of metal cations resulted in the absorption of the cation to the biomass with the concomitant disappearance of the soluble metal from solution. The biosorption capacity of the organism is summarized in the table below:

Metal ion	Biosorption capacity (μmol metal/g dry cell weight)
Ag(I)	680
Tl(I)	270
Mn(II)	330
Fe(II)	520
Co(II)	400
Ni(II)	400
Cu(II)	410
Zn(II)	350
Pd(II)	490
Cd(II)	360
Pb(II)	320
Cr(III)	370
Y(III)	170
U(VI)	290

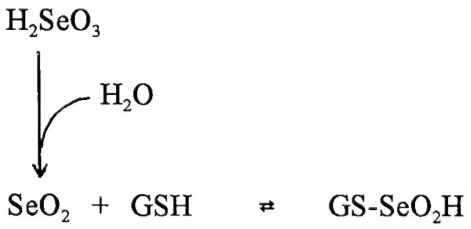
Identification, separation, purification, and reconstitution of the minimum cellular components necessary for metal transformation in strain OR-02

(i) Mercury - A mercuric reductase was purified to electro-phoretic homogeneity from cell-free extracts of OR-02 grown in the presence of 100 μM Hg(II). The purified enzyme was a soluble dimer comprised of identical subunits of 60,000 daltons. The enzyme contained one FAD per subunit and catalyzed the NADPH-dependent reduction of Hg(II) to Hg(0) in the presence of excess exogenous thiols. With 2-mercaptoethanol as the exogenous thiol, the reduction of mercuric ions obeyed Michaelis-Menten saturation kinetics with values of K_m for NADPH and RSHgSR of 15 and 6.0 μM, respectively, and a turnover number of 270 min^{-1}. The structural and functional properties of the mercuric reductase from OR-02 were thus similar to those of analogous enzymes from other bacteria. The mercuric reductase-dependent oxidation of NADPH was entirely specific for mercuric ions. No enzyme-dependent oxidation of NADPH could be detected in the presence of any of the other 7 soluble metal precipitated by this bacterium.

(ii) Selenium - A clue to the molecular mechanism of intracellular selenite reduction was afforded by the bacterial growth studies. When OR-02 was cultured in nutrient broth containing 40 mM selenite, an abundant red precipitate was visible evidence of the microbial-dependent reduction of the selenite. Strain OR-02 also showed abundant growth in nutrient broth containing 2.0 mM buthionine sulfoximine (BSO). BSO is an irreversible inhibitor of γ-glutamylcysteine synthetase and has been widely used to selectively inhibit glutathione biosynthesis without adversely affecting amino acid biosynthesis in general. When both BSO and selenite were present simultaneously, the bacterium would not even grow, much less precipitate elemental selenium. This growth experiment underscored the importance of glutathione in the protective mechanism(s) adopted by the OR-02 in response to toxic levels of selenite.

Cell-free extracts of OR-02 contained millimolar levels of reduced glutathione (GSH) and detectable glutathione reductase activity. When selenite was rapidly mixed with an excess of GSH in a stopped flow spectrophotometer, the absorbance of the resulting solution underwent three sequential changes consistent with the following chemical changes (data not shown):

First phase



Second phase

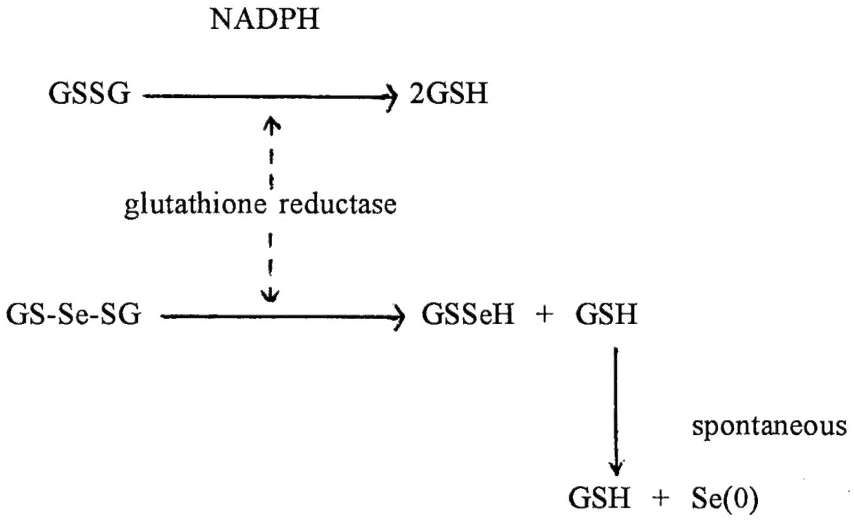
$$GS\text{-}SeO_2H + GSH \rightleftharpoons GS\text{-}Se(O)\text{-}SG + H_2O$$

Third Phase

$$GS\text{-}Se(O)\text{-}SG + GSH \rightleftharpoons GS\text{-}Se\text{-}SG + GSOH$$

$$GSOH \xrightarrow{GSH} GSSG + H_2O$$

The bis(glutathio)selenium, GS-Se-SG, formed as a result of the spontaneous, uncatalyzed reactions introduced above, can then serve as a reactive substrate analog for glutathione reductase,



resulting in the generation and precipitation of elemental selenium. When NADPH, GSH and selenite were incubated with the soluble fraction of cell-free extracts of OR-02 grown in the presence of selenite, timely precipitation of red, elemental selenium was observed. The omission of NADPH or GSH, treatment of the cell-free extract by proteases or boiling, or the addition of aromatic arsenicals (which inhibit glutathione reductase) all served to strongly inhibit the appearance of red precipitate. Furthermore, the specific activity of the glutathione reductase activity was observed to be 5- to 10-fold higher in extracts prepared from selenite-grown cells when compared to that in extracts prepared from cells grown in the absence of selenite.

The hypothesis constructed from the studies summarized above is that the generation of elemental selenium occurs as a consequence of the glutathione reductase-dependent reduction of the bis(glutathio)selenium that forms spontaneously when selenite and GSH are present together. A manuscript that describes these results is in preparation for submission to the Journal of Biological Chemistry, as indicated below.

(iii) Tellurium - When OR-02 was grown in nutrient broth containing up to 10.0 mM tellurite, growth of the bacterium occurred concomitantly with the precipitation of the black elemental form of tellurium, Te(0). A series of experiments analogous to those described above were conducted in which tellurite was substituted for selenium. The cell growth experiment with BSO outlined above produced the same conclusion when tellurite was substituted for selenite. Furthermore, we observed that tellurite and GSH reacted in a manner similar to that described above to generate bis(glutathio)tellurium, GS-Te-SG. The current working hypothesis is that the generation of elemental tellurium occurs as a consequence of the glutathione reductase-dependent reduction of the bis(glutathio)tellurium that forms spontaneously when tellurite and GSH are present together.

It is anticipated that these experiments featuring the reduction of tellurite will eventually lead to a separate publication.

(iv) Lead - When OR-02 was grown in nutrient broth containing up to 3.0 mM lead nitrate, growth of the bacterium occurred concomitantly with the appearance of a black precipitate. A combined SEM and energy dispersive X-ray analysis was performed on bacterial cells cultured in the presence of lead nitrate. In contrast to the results obtained with selenite, growth of OR-02 in the presence of Pb(II) led to the appearance of dark, electron-dense bodies **outside** of the bacterial cell. These dark extracellular bodies contained all of the detectable lead; lead could not be detected by energy dispersive X-ray spectrophotometry either inside the cell or on the plasma membrane. Furthermore, the presence of BSO had little effect on the growth and lead-transformation activity of the organism in the presence of Pb(II). It has thus become evident that discreet molecular mechanisms exist for the bacterial transformations of Pb(II) and Se(IV), not an entirely unexpected conclusion.

(v) Chromium - Cells of strain OR-02 that were adapted for the reduction of chromate to trivalent chromium(III) were disrupted and examined for a cell-free chromate reductase activity. The only cell-free, pyridine nucleotide-dependent reduction of chromate that could be detected was located in the membrane fraction of cells that had been disrupted by sonic oscillation. Both NADH and NADPH supported chromate reduction. The pH optimum was 7.5 with an apparent Km for chromate of 100 μM. The membrane-bound chromate reductase activity was quite labile and lost 70-80% of the original activity after 24 hours at 4°C. The enzyme(s) responsible for chromate reduction was not investigated further because of this discouraging stability problem.

Related observations and experiments

Bacteria from other toxic waste sites catalyze metal transformations similar to those of strain OR02.

A collaboration was initiated with the laboratory of Dr. Larry Barton at New Mexico State University in Albuquerque, NM. In addition to the SEM experiments discussed above, we participated in the isolation and preliminary characterization of metal-transforming bacteria from chromium(principally chromate)-contaminated soil at Sandia and Los Alamos National Laboratories. Several isolates were discovered that electrochemically reduced chromate and mercuric ions. In addition, a handful of these isolates also transformed soluble lead in a manner similar to that of strain OR-02.

Acquisition of these new isolates created the possibility of investigating particular metal-transformation activities in more than one organism. The knowledge accumulated within each stable of organisms that appear to share a fundamentally common pathway of metal transformation may provide opportunities to study a particular portion of the pathway in some of the members that proves to be intractable in other members. For example, a key protein in the pathway that is extremely difficult to isolate from one organism may prove to be easily obtained in good yield from another. On the other hand, detailed comparisons among the groups of organisms that appear to express different mechanisms and pathways for selected metal transformations may provide the opportunity to deduce the advantages and disadvantages of each. In terms of the the eventual application of this basic knowledge to bioremediation problems, it should be emphasized that each metal-transforming organism was isolated from a hazardous waste environment. Although strain OR-02 has received the most attention in the laboratory, that does not mean that it will prove to be the predominant or most useful organism in any or all of the eventual bioremediation processes that may be developed. The need for basic information regarding the metal transformation activities of all of these organisms is evident.

Some of the metal transformation activities of strain OR-02 were mobilized to other bacteria.

A second collaboration was initiated between this laboratory and that of Dr. Julius Jackson, a molecular geneticist at Michigan State University. Dr. Jackson used plasmid DNA derived from strain OR-02 to transform various metal-resistance phenotypes into a recipient strain of *E. coli*. He supplied this laboratory with stable *E. coli* transformants that either (i) reduced Se(IV) to elemental selenium, (ii) reduced Hg(II) to elemental mercury, or (iii) immobilized Pb(II) as a brown-black precipitate. The acquisition of these transformants broadens the opportunities to study the molecular mechanism(s) of each metal transformation. The ability to transfer metal-immobilization phenotypes into other bacteria could also permit the eventual transformation of indigenous bacterial populations already adapted to and inhabiting selected heavily polluted sites.

Certain facultative anaerobic bacteria will electrochemically reduce insoluble metal oxides.

A third collaboration was initiated with Dr. Patricia Rusin, senior microbiologist with Metallurgical and Biological Extractions, Inc., Tucson, AZ. Dr. Rusin isolated over 300 bacteria from Crystal Hill, CA, and Hardshell, AZ, mine samples for their ability to electrochemically reduce and solubilize manganese dioxide under anaerobic conditions. Isolate D1 demonstrated a capacity to reduce manganese dioxide superior to that of any other organism described to date. Furthermore, strain D1 also exhibited the facile electrochemical reduction of insoluble iron oxides. This bacterium and others like it hold great promise for the bioremediation of a variety of insoluble metal oxides. Indeed, at the PI's suggestion, Dr. Rusin spent 2 weeks doing collaborative experiments with Dr. James Brainard at Los Alamos National Laboratory in New Mexico. They were able to demonstrate the facile bacterial-dependent electrochemical reduction of insoluble plutonium dioxide [Pu(IV)] to soluble Pu(III). It may thus be possible to devise strategies to remediate plutonium-containing materials based on the bacterial-dependent mobilization of the insoluble radionuclide into the aqueous phase. This

PUBLICATIONS

(i) Published - five

R.C. Blake II, D.M. Choate, S. Bardhan, N. Revis, L.L. Barton, and T.G. Zocco (1993) "Chemical Transformation of Toxic Metals by a *Pseudomonas* Strain from a Toxic Waste Site", *Environ. Tox. Chem.* **12**, 1365-1376

L.L. Barton, H.E. Nuttall, W.C. Lindemann, and R.C. Blake II (1993) "Biocolloid Formation: An Approach to Bioremediation of Toxic Metal Wastes" in *Handbook of Bioremediation* (D.L. Wise and D.J. Trantolo, Eds.) Marcel Dekker, New York, pp. 481-496

R.C. Blake II, and G. Bowers-Irons (1993) "Bioremediation of Metal Arsenide Wastes" in *Biocorrosion and Biofouling: Metal/Microbe Interactions* (H.A. Videla, Z. Lewandowski, and R.W.Lutey, Eds.) Buckman Laboratories International, Inc., Memphis, TN, pp. 162-170

L.L. Barton, F.A. Fekete, L.O. Sillerud, C.J. Pigg, and R.C. Blake II (1994) "Application of biotechnology in management of industrial wastes containing toxic metals", *Radioactive Waste Management and Environment Restoration* **18**,13-25

Y.S. Gao, and R.C. Blake II (1997) "Separation of bacteria by sedimentation field flow fractionation", *J. Microcolumn Sep.*, in press

(ii) Submitted - none

(iii) In preparation - two

R.C. Blake II, J. Jackson, G. Howard, and Y.S. Gao, "On the transformation of metals by bacteria. Electrochemical reduction of selenite to elemental selenium by Xanthomonas maltophilia", to be submitted to *J. Biol. Chem.*

R.C. Blake II, " Rapid mixing kinetic analysis of the reaction of selenite with excess reduced glutathione", to be submitted to *J. Biol. Chem.*

PROFESSIONAL PERSONNEL

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